# ATTACHEMENT II – PROTOCOL

ECOLAB
Study Identification Number 1200077

## REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

**STUDY TITLE: Aqualogic Germicidal Spray Disinfection Efficacy at 10 minutes**

**EPA REG. NO.: 1677-**

**STUDY IDENTIFICATION NUMBER: 1200077**

## PROPOSED STUDY INITIATION/COMPLETION DATES

**Initiation** September 10, 2012

**Completion** October 31, 2012

## DESCRIPTION OF STUDY OBJECTIVE

**Aqualogic** (EPA Registration No. 1677- ) will be tested according to Ecolab Microbiological Services SOP Method MS010-21; *Germicidal Spray Products as Disinfectants* against *Enterococcus faecalis* – VRE ATCC 51299 and *Escherichia coli* 0157:H7 ATCC 43895. The test systems will be exposed to the test substance for 10 minutes at room temperature (15-30°C) when diluted to 0.0660% free available chlorine in sterile laboratory purified water per the Confidential Statement of Formula (CSF). The actual dilutions which will be performed for the test substance use-solutions will be determined subsequent to the chemical quality verification to deliver the required level of active ingredient. The test substance will be challenged by the addition of 5% fetal bovine serum to the test systems. The test substance will be applied to the carriers at a distance of 6-8 inches for 3 sprays. AOAC Method 961.02, Chapter 6 Disinfectants, 2009 was the test method utilized in making the disinfectant claim.

ECOLAB
Study Identification Number 1200077

## TEST SUBSTANCE IDENTIFICATION

**Test Substance Name: Aqualogic**

**Batch Identification:**

1. **051512DT**
2. **052912DT**

The use-solution chemical quality verification will be performed on both batches.

An aliquot of each test substance batch will be retained in the retention cabinet at ECOLAB Schuman Campus until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in ECOLAB Microbiological Services laboratory until disposed.

## QUALITY ASSURANCE UNIT MONITORING

The protocol, chemical quality verification in-life inspection, chemical quality verification in-life data audit, pesticide efficacy in-life and final report are proposed to be inspected by the ECOLAB Quality Assurance Unit (QAU) in accordance with their current Standard Operating Procedures. The following proposed ECOLAB QA inspections are for planning purposes only and may change. ECOLAB QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in ECOLAB QA inspections from those proposed below will not require revision of this protocol.

### A. Proposed QAU Monitoring

| |
|---|
| Protocol Audit |
| Chemical Quality Verification In-Life Inspection |
| Chemical Quality Verification Data Audit |
| Pesticide Efficacy In-Life Inspection |
| Final Report Audit |

ECOLAB
Study Identification Number 1200077

## CHEMICAL QUALITY VERIFICATION

### A. Proposed Experimental Initiation/Termination Dates

Experimental Initiation Date: September 12, 2012

Experimental Termination Date: September 12, 2012

### B. Method

Chemical analysis will be performed on each test substance batch to determine the concentration of the active ingredient. Chemical analysis will also be performed on the test substance use-solutions. The use-solution preparation will be documented in the raw data.

The test substance is a ready-to-use product that will be diluted at or below the lower certified limit for the active ingredient. The following calculation will used to determine the amount of test substance in a 500 g use-solution diluted to 660 ppm (or 0.0660%) free available chlorine:

ppm at LCL = (% LCL/100) (specific gravity) $10^6$ =
ppm at LCL = (0.0660%/100) X (0.999) X $10^6$ = 659 ppm

Amount of test substance in 500 g use-solution to result in 659 ppm =

$$\frac{659 \text{ ppm} \times 100 \times 500}{(\%\text{ active})\ 10^6}$$

Note: It is acceptable to change the desired volume of the use-solution in the calculation above to either make more or less. The weights used to prepare the use-solution may vary by +/- 0.03 grams from the weights calculated.

In order to prepare the use-solution using weight to weight measurements, the specific gravity was incorporated into the calculations resulting in 659 ppm (or 0.0659%) free available chlorine as the lower certified limit.

The chemical quality verification will be performed by the Analytical Lab using the method listed below. The method has been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution samples.

QATM-007; Available Chlorine
Available chlorine content is determined by reduction of the chlorine to chloride by iodide ion. The iodine liberated by this reaction is then determined by titration with sodium thiosulfate, either manually or potentiometrically with an automatic titrator.
The most current QATM will be used during the course of this study for the chemical and physical analysis.

ECOLAB
Study Identification Number 1200077

## C. Interpretation of Results

The concentration of the active ingredient in the test substance batches will be judged acceptable for pesticide efficacy testing if within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

| Active Ingredient | CSF Lower Certified Limit | CSF Upper Certified Limit |
|---|---|---|
| Free Available Chlorine* | 0.0660% | 0.1030% |

*The equivalent weight of NaOCL (sodium hypochlorite) to the equivalent weight of $Cl_2$ (Chlorine) is 37.2/35.5 = 1.05. Dividing the sodium hypochlorite concentration by the ratio of the equivalent weight of sodium hypochlorite to the equivalent weight of chlorine results in the free available chlorine concentration.

The concentration of the active ingredients in the test substance diluted to the lower certified limit (test substance use-solution) will be judged acceptable for pesticide efficacy testing if within the acceptance limit of 0.0594 – 0.0726% available chlorine.

After diluting the ready-to-use test substance to the lower certified limit of 0.0660% free available chlorine, the nominal concentration of the active ingredient is <1.0%. Therefore, the Calculated Lower Acceptance Limit and Calculated Upper Acceptance Limit for available chlorine will be expanded to accommodate method variability and suitable rationale. The expanded ranges are based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for available chlorine
= [0.0660% - (0.0660 x 0.1)] = 0.0594%
Calculated Upper Acceptance Limit for available chlorine
= [0.0660% + (0.0660 x 0.1)] = 0.0726%

The chemical quality verification raw data will be reported in the final report of this study.

ECOLAB
Study Identification Number 1200077

**PESTICIDE EFFICACY TESTING**

**A. <u>Proposed</u> Experimental Start/Termination Dates**

| | |
|---|---|
| Experimental Start | September 12, 2012 |
| Experimental Termination | September 17, 2012 |

**B. Methods**

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

| Method Number | Method Name |
|---|---|
| MS002-16 | *Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims* |
| MS010-21* | *Germicidal Spray Products as Disinfectants* |
| MS088-18 | *Test Substance Use-Solution Preparation for Analysis* |
| MS111-04** | *Antibiotic Susceptibility Tests* |

*MS010 will be followed with the following exception:
The test system culture will be uniformly spread over the carrier to within approximately 1/8 inch of the edges.
**Antibiotic susceptibility testing will be performed on *Enterococcus faecalis* – VRE ATCC 51299.

**<u>Test Method Requirement and Test System Justification</u>**

The following apply when determining the effectiveness of a disinfectant on supplemental organism; 10 carriers are required on each of two samples, representing different batches. The organisms to be tested in this study are *Enterococcus faecalis* – VRE ATCC 51299 and *Escherichia coli* 0157:H7 ATCC 43895. The AOAC Germicdal Spray Products Test will be used for the above stated organisms based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 Disinfectants for Use on Hard Surfaces –Efficacy Data Recommendations March 12, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000 General considerations for Public Health Uses of Antimicrobial Agents March 12, 2012 applies to this study.

**<u>Test Method Justification</u>**

Ecolab Microbiological Services SOP MS010-21; *Germicidal Spray Products as Disinfectants* will be the test method utilized in this study.

ECOLAB
Study Identification Number 1200077

**Statement of Proposed Statistical Method**

None

**Test Systems and Identification**

The test systems which will be utilized for this procedure are *Enterococcus faecalis* – VRE ATCC 51299 and *Escherichia coli* 0157:H7 ATCC 43895. Identification will be performed by observing the colony morphology and performing a Gram stain.

**Organic Soil Load**

5% Fetal Bovine Serum

**Test Substance Diluent**

Sterile laboratory purified water

**Test Substance Concentration**

Antimicrobial efficacy testing will be performed with **Aqualogic** diluted to at or below the lower limit of 660 ppm.

| Active Ingredient | CSF Lower Certified Limit | CSF Upper Certified Limit |
|---|---|---|
| Available Chlorine | 0.0660% | 0.1030% |

The dilution procedure is based on results of the Chemical Quality Verification. To achieve dilution of the ready-to-use test substance to at or below the lower certified limit of available chlorine, the test substance batches will be prepared based on the available chlorine results and documented in the raw data. The following calculations will be used to determine the dilution procedure for each test substance batch to result in the lower certified limit of available chlorine.

ppm at LCL = (% LCL/100) (specific gravity) $10^6$ =
ppm at LCL = (0.0660%/100) X (0.999) X $10^6$ = 659 ppm

Amount of test substance in 500 g use-solution to result in 659 ppm =

$$\frac{659 \text{ ppm} \times 100 \times 500}{(\%\text{ active})\ 10^6}$$

Note: It is acceptable to change the desired volume of the use-solution in the calculation above to either make more or less. The weights used to prepare the use-solution may vary by +/- 0.03 grams from the weights calculated.

ECOLAB
Study Identification Number 1200077

**Test Surface**

Microscope slides, non-corrosive, 25 x 25 mm (1" x 1")

**Exposure Time/Temperature**

The test systems will be exposed to the test substance for 10 minutes at room temperature (15-30°C)

**Spray Distance and Number of Trigger Sprays**

The test substance will be applied by spraying the carriers at a distance of 6-8 inches for 3 sprays

**Neutralizer/Subculture Medium**

Brain Heart Infusion Broth with 0.5% Sodium Thiosulfate will be used *Escherichia coli* 0157:H7 ATCC 43895

Brain Heart Infusion Broth with 0.1% Sodium Thiosulfate + 0.7 g Lecithin per liter + 5 g Tween 80 per liter will be used for *Enterococcus faecalis* – VRE ATCC 51299.

**Plating Medium**

Brain Heart Infusion Agar

**Incubation Time/Temperature**

Tubes and plates will be incubated for 48 ± 4 hours at 35 ± 2°C

**Test Controls**

The following controls will be incorporated with the test procedure for each test system:

a. Average Volume/Weight Delivered per Carrier
b. Carrier Enumeration
c. Positive Control
d. Negative Control
e. Diluent Sterility
f. Fetal Bovine Serum Sterility
g. Neutralization Confirmation
h. Test System Purity

Details on each of the above controls can be found in Ecolab SOP MS010-21 located in Protocol Appendix.

ECOLAB
Study Identification Number 1200077

**Verification of Test System in Positive Subculture Tubes**

All positive tubes from the test will be subcultured to Tryptic Soy Agar with 5% Sheep's Blood. The colony morphology observed will be compared to the typical colony morphology of the test system for verification of the test system in positive subculture tubes.

**Interpretation of Test Results**

The performance standard for a disinfectant requires the product to kill the test organism on at 10 out of 10 carriers to provide significance at the 95 % confidence level.

## DATA RETENTION

Following completion of the study, an exact copy of the final report and the original raw data and protocol will be transferred to ECOLAB Archives at the ECOLAB Schuman Campus in Eagan, MN or an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

## TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the retention cabinet at the ECOLAB Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

## GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

ECOLAB
Study Identification Number 1200077

- **Name and Address of Sponsor**

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- **Name and Address of Testing Facility**

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Sponsor — Date: 09/10/2012

Study Director — Date: 9/10/12

ECOLAB
Study Identification Number 1200077

## PROTOCOL APPENDIX

**Microbiological Services (MS) Methods:**

| | | |
|---|---|---|
| MS002-16 | *Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims* | Pages 1-3 |
| MS010-21 | *Germicidal Spray Products as Disinfectants* | Pages 1-9 |
| MS088-18 | *Test Substance Use-Solution Preparation For Analysis* | Pages 1-6 |
| MS111-04 | *Antibiotic Susceptibility Tests* | Pages 1-7 |

Standard Operating Procedure

Ecolab Controlled Document

**ECOLAB**
**MICROBIOLOGICAL SERVICES**

**TITLE:** **Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims**

**NUMBER:** **MS002-16**

**EFFECTIVE:** **09/01/12**

## 1.0 PURPOSE

The addition of organic soil to a test procedure is necessary to allow for the one-step cleaner disinfectant or cleaner sanitizer claim.

## 2.0 SCOPE

2.1 An antimicrobial product that bears the label claim of a one-step cleaner disinfectant or cleaner sanitizer, or one intended to be effective in the presence of organic soil, must be tested for efficacy by the appropriate method(s) which have been modified to include a representative soil such as 5% fetal bovine serum (e.g. blood serum).

## 3.0 STORAGE & HANDLING INSTRUCTIONS FOR FETAL BOVINE SERUM

3.1 Fetal bovine serum is to be stored at ≤ -10°C and used prior to the expiration date on the bottle label.

3.2 Fetal bovine serum may be thawed and dispensed into vials. Thawed fetal bovine serum may be stored at 2 – 8°C for up to two months from the date thawed.

3.3 Document the following information on Form 3070:

- Fetal Bovine Serum manufacturer
- Lot number
- Bottle identification
- Bottle expiration date
- Thawed date
- Dispensed date
- New expiration date
- Initial & Date

## 4.0 TEST REQUIREMENTS

4.1 A suggested procedure to simulate in-use conditions where the antimicrobial agent is intended to treat dry inanimate surfaces with an organic soil load involves contamination of the appropriate carrier surface with each test microorganism

EXACT COPY

**TITLE:** **Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims**

**NUMBER:** **MS002-16**

culture containing 5% (v/v) fetal bovine serum (e.g. 19 mL test system and 1 mL fetal bovine serum) prior to the specified carrier-drying step in the method.

**Note:** The organic soil should be added to the test system suspension prior to inoculation of the test surface or test substance.

4.2 A 5% level of organic soil is considered appropriate for simulating lightly or moderately soiled surfaces. When the surface to be treated is heavily soiled, a cleaning step must be recommended prior to application of the antimicrobial. The effectiveness of antimicrobial agents must be demonstrated in the presence of a specific organic soil at an appropriate concentration level according to a specific label claim. Additional organic soil does not need to be incorporated if at least 5% blood serum is already present.

4.3 If an antimicrobial product has a soap scum claim, an appropriate method of simulation would be to add a 0.005% concentration of sodium stearate. A 1:20 dilution of a 0.1% solution of sodium stearate is made into the culture inoculum for a final concentration of 0.005%.

4.4 An alternate method of introducing the organic soil where the antimicrobial agent is not tested against a dry inanimate surface involves adding 5% organic soil to the use-solution of a product prior to inoculation with the test system [e.g. 4.75 mL use-solution + 0.25 mL fetal bovine serum before adding 0.5 mL of the required level ($5 \times 10^6$/mL) of conidia].

## 5.0 REGULATORY EFFICACY REPORTS

5.1 The level and type of organic soil must be stated in the protocol, raw data and the final efficacy report. The method of incorporation of organic soil must also be stated (e.g. 5% fetal bovine serum added to the test system suspension for the inoculation of the test surface or test substance).

5.2 The Certificate of Analysis for the fetal bovine serum shall be included in the study file.

## 6.0 RECORD MAINTENANCE

6.1 Records will be stored in the Test System binder located in Microbiological Services. Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2012 will be archived by March of 2014. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

JR 9/10/12

**TITLE:** **Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims**

**NUMBER:** **MS002-16**

## 7.0 RELATED FORMS

7.1 Form 3070: Fetal Bovine Serum

## 8.0 REFERENCES

8.1 US EPA OCSPP 810.2000 General Considerations for Public Health Uses of Antimicrobial Agents, March 12, 2012

## 9.0 MOST RECENT REVISION SUMMARY

Split Thawed/dispensed date into two separate bullets in 3.3. Added that additional organic soil does not need to be incorporated if at least 5% blood serum is already present to 4.2. Updated example years in 6.1. Changed the reference in section 8.0.

Prepared by: Laurinda Holen Date: 8/7/12

Quality Assurance: Sherri St. Clair Date: 07 Aug 2012

Management: [signature] Date: 08 Aug 2012

JN 9/10/12

**ECOLAB**
**MICROGIOLOGICAL SERVICES**

**TITLE:** **Germicidal Spray Products as Disinfectants**

**NUMBER:** **MS010-21**

**EFFECTIVE:** **09/01/12**

## 1.0 PURPOSE

To determine the effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces.

## 2.0 CULTURE MEDIA

2.1 AOAC Nutrient Broth
2.2 AOAC Synthetic Broth, containing 0.1 mL 10% Dextrose solution per 10 mL broth tube
2.3 Nutrient Agar
2.4 Sabouraud Dextrose Agar
2.5 Sabouraud Dextrose Broth
2.6 Glucose Agar
2.7 Other culture media as appropriate for test system

## 3.0 SUBCULTURE MEDIA

3.1 Letheen Broth
3.2 Fluid Thioglycollate Medium
3.3 Sabouraud Dextrose Broth
3.4 Sabouraud Dextrose Broth with 0.07 % lecithin & 0.5% polysorbate 80 (tween 80)
3.5 Glucose Broth
3.6 CTA Medium (Cystine Tryptic Agar)
3.7 MacConkey agar
3.8 Mannitol Salt agar
3.9 Pseudosel agar
3.10 Other media as appropriate for test system

## 4.0 APPARATUS

4.1 Test tubes: 20 × 150, 25 × 150 and 32 × 200 mm glass test tubes
4.2 Petri dishes: Glass, containing two layers of Whatman No. 2 filter paper, or equivalent
4.3 Test tube racks: Any convenient style capable of holding 20 × 150, 25 × 150 or 32 × 200 mm test tubes

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**TITLE: Germicidal Spray Products as Disinfectants**

**NUMBER: MS010-21**

4.4 Transfer loops: Platinum-rhodium with a 4 mm inside diameter, or plastic disposable transfer loops may be used
4.5 Micropipetter and sterile pipette tips to deliver 0.01 mL
4.6 Microscope coverslips (carrier): non-corrosive, 25 × 25 mm (1" × 1") glass coverslips
4.7 Microscope slides, non-corrosive, 25 × 25 mm (1" × 1")
4.8 Metal forceps: Sharp points

## 5.0 REAGENTS

5.1 Diluent (refer to MS008 if preparing synthetic hard water)
5.2 Blood serum or other appropriate organic load, if applicable (refer to MS002)
5.3 0.85% Saline Solution
5.4 Triton X-100

## 6.0 TEST SYSTEM PREPARATION (Culture Preparation)

6.1 For *Staphylococcus aureus* ATCC 6538 and *Salmonella enterica* ATCC 10708 follow steps listed below:

6.1.1 A minimum of three consecutive transfers but less than 15 total transfers in AOAC Nutrient Broth (AOAC Synthetic Broth may also be used for *Staphylococcus aureus* and *Salmonella enterica*) need to be made before using to inoculate for testing.

6.1.2 If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers.

6.1.3 If two or more transfers are missed, repeat with the three consecutive transfers.

6.1.4 Transfers of *Staphylococcus aureus* and *Salmonella enterica* are made on a 24 hour schedule. The last consecutive transfer used to inoculate for the test should be a 24 ± 4 hour test system.

6.1.5 Inoculate a sufficient number of culture media broth tubes for the test. Broth tubes should contain 20 mL of appropriate media. Incubate for 48 – 54 hours at 35 ± 2°C.

6.1.6 Vortex the 48 – 54 hour culture for three to four seconds to mix. Allow culture to stand for ten minutes. Remove the upper portion (approximately 15 mL) of the culture and use for the test.

6.1.7 Add soil challenge (blood serum and/or soap residue) to the culture if required by the test protocol (refer to MS002). Vortex to mix.

**TITLE:** **Germicidal Spray Products as Disinfectants**

**NUMBER:** **MS010-21**

6.2 For *Pseudomonas aeruginosa* ATTC 15442

6.2.1 A minimum of three consecutive transfers but less than 15 total transfers in AOAC Nutrient Broth (AOAC Synthetic Broth may also be used) need to be made before using to inoculate for testing.

6.2.2 If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers.

6.2.3 If two or more transfers are missed, repeat with the three consecutive transfers.

6.2.4 Transfers of *Pseudomonas aeruginosa* are made on a 24 hour schedule. The last consecutive transfer used to inoculate for the test should be a 24 ± 4 hour test system.

6.2.5 Inoculate a sufficient number of culture media broth tubes for the test. Broth tubes should contain 20 mL of appropriate media. Incubate for 48 – 54 hours at 35 ± 2°C.

6.2.6 After incubation do not shake the culture but decant, aspirate or use a loop to aseptically remove the pellicle from the liquid culture. Cultures in which the pellicle has been disturbed may not be used in tests.

6.2.7 Vortex the 48 – 54 hour culture for three to four seconds to mix. Allow culture to stand for ten minutes. Remove the upper portion (approximately 15 mL) of the culture and use for the test.

6.2.8 Add soil challenge (blood serum and/or soap residue) to the culture if required by the test protocol (refer to MS002). Vortex to mix.

6.3 Other test systems may be used in this procedure. Modification of culture medium, incubation time, and incubation temperature may be necessary.

6.4 Visually inspect culture and <u>do not</u> use if culture looks atypical (e.g. contains chunks or particles).

6.5 For *Trichophyton mentagrophytes* ATCC 9533 and *Aspergillus niger* ATCC 6275

**Note:** Do not use culture that has been kept at or above room temperature for more than ten days as the source of inoculum for culture.

6.5.1 Inoculate the center of Glucose Agar or Sabouraud Dextrose Agar petri dishes at 26 ± 2°C for 10 – 15 days.

**TITLE:** **Germicidal Spray Products as Disinfectants**

**NUMBER:** **MS010-21**

6.5.2 Remove the mycelial mats from the surface of agar plates. Mats may be removed by adding 2 – 5 mL physiological saline (0.85% NaCl) to each plate, scraping the mat with an appropriate tool and then transferring suspension to a sterile glass tissue grinder or sterile Erlenmeyer flask containing glass beads. Add saline to the tissue grinder or flask and macerate by grinding (tissue grinder) or shaking flask thoroughly.

6.5.3 Filter the suspension through two layers of sterile cheesecloth to remove hyphal elements.

6.5.4 Add 0.02 mL Triton X-100 per 10 mL test system suspension to facilitate spreading on the glass slide.

6.5.5 Estimate the density of the conidial suspension by performing a plate count on the suspension using serial dilutions and pour or spread plate technique.

6.5.6 Incubate the plates at 26 ± 2°C for three to five days or until there is sufficient growth to count.

6.5.7 Standardize the conidial suspension as needed by diluting stock spore suspension (or concentrating by centrifugation, then diluting) with physiological saline so that it contains approximately 5 x $10^6$ conidial/mL. The suspension may be stored at 2 – 8°C for ≤ four weeks.

## 7.0 CARRIER PREPARATION

7.1 Either 1" × 1" microscope slides or microscope coverslips may be used for the test. One carrier type must be used for the entire test.

7.2 Discard any carriers that are damaged.

7.3 Before sterilization, carriers must be rinsed in 95% ethanol and then rinsed in deionized water. This removes any oil or films that may be on the carriers.

7.4 Dried carriers are autoclave sterilized in glass petri dishes matted with two pieces of Whatman No. 2. or equivalent, filter paper. One carrier is used per petri dish. Carriers may be sterilized in a hot air oven for two hours at 180°C or in an autoclave steam cycle for 20 minutes with a drying cycle.

## 8.0 TEST SUBSTANCE PREPARATION

8.1 Prepare (disinfectant) test substance use solutions ≤ three hours prior to use, unless otherwise specified. Prepare test substance dilution in a sterile volumetric flask according to instructions (e.g. if a 1:128 dilution is required, add 1 part test

**TITLE: Germicidal Spray Products as Disinfectants**

**NUMBER: MS010-21**

material + 127 parts diluent). If the test substance requires dilution, use ≥ 1.0 mL or ≥ 1 g of test substance to prepare the use solution.

## 9.0 INOCULATION OF CARRIERS

9.1 Inoculate a sterile 1" × 1" square carrier in a glass petri dish with 0.01 mL of test system suspension. There should be one carrier in each petri dish. Vortex the test system suspension periodically during inoculation of the carriers.

9.2 Spread the culture uniformly over the entire carrier.

9.3 Record relative humidity of the incubator before the first set of carriers is dried.

9.4 Cover the petri dish and dry 30 – 40 minutes at 35 ± 2°C.

## 10.0 OPERATING PROCEDURE

10.1 Spray inoculated carrier for specified time and distance at regular intervals (refer to 11.1 for more information). Hold each carrier for the exposure time then drain off excess test substance and aseptically transfer each carrier at regular intervals to individual 32 × 200 mm test tubes containing 20 mL of appropriate subculture medium using sterile forceps. Shake tubes thoroughly.

10.2 Both the spraying and transfer to subculture medium should be performed ± five seconds of the actual transfer. For test substance with ≤ one minute exposure time, sprays and transfers need to be completed within ± three seconds.

10.3 If broth appears cloudy after 30 minutes, make a second subculture to fresh medium (secondary subculture).

10.4 Incubate all bacterial tubes (primary and secondary subcultures) for 48 ± 4 hours at 35 ± 2°C. *Trichophyton mentagrophytes* and *Aspergillus niger* are incubated for 10 – 15 days at 26 ± 2°C.

## 11.0 CONTROLS

11.1 Determination of average volume/weight delivered during product application.

11.1.1 Determine the average volume applied by spraying a petri dish at least five times with the same applicator and test substance used during efficacy testing.

11.1.2 Place a petri dish on a balance and tare. Spray the petri dish for the specified time or for the required number of trigger squeezes at the appropriate distance. Record the weight.

**TITLE: Germicidal Spray Products as Disinfectants**

**NUMBER: MS010-21**

11.1.3 Repeat this step at least four times, for a total of ≥ five weights.

11.1.4 Average the weights to determine the average volume/weight per carrier.

**Note:** All controls should be incubated at 35 ± 2°C for 48 ± 4 hours for bacteria and 26 ± 2°C for 10 – 15 days for fungi.

11.2 Carrier Enumeration (CFU/Carrier)

11.2.1 Perform count for each test system used in testing. A minimum of three carriers per set dried, per test system should be enumerated.

11.2.2 Place each dried inoculated carrier into 20 mL of subculture broth.

**Note:** For each batch of carriers to be enumerated, this step must be performed after all the test carriers in the batch have been exposed to the test substance.

11.2.3 Vortex the carrier/subculture medium mixture vigorously for approximately 30 seconds. Alternately, the carrier/subculture medium mixture may be sonicated for 30 seconds to five minutes.

11.2.4 Prepare serial dilutions in Phosphate Buffer Dilution Water (PBDW) and plate in duplicate $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions using standard plating procedures. Other dilutions may be plated as appropriate.

11.2.5 A minimum of $10^4$ CFU/carrier is required for a valid test. The average CFU/carrier is the average CFU/mL × 20 mL/carrier.

11.3 Viability Controls

11.3.1 Positive control

11.3.1.1 Place two dried inoculated carriers into separate tubes containing 20 mL of subculture broth. Positive growth in both tubes is required for a valid test.

11.3.2 Negative control

11.3.2.1 Place one negative carrier into 20 mL of substance broth. No growth in the tube is required for a valid test.

**TITLE: Germicidal Spray Products as Disinfectants**

**NUMBER: MS010-21**

11.4 Test Substance Diluent

11.4.1 Subculture 1 mL of the diluent into a subculture medium test tube. No growth in the test tube is required for a valid test.

11.5 Blood Serum Sterility Control, if applicable.

11.5.1 Subculture 1 mL of Blood Serum into subculture medium tube. No growth in the tube is required for a valid test.

11.6 Sodium Stearate Sterility Control, if applicable

11.6.1 Subculture 1 mL of Sodium Stearate into subculture medium tube. No growth in the tube is required for a valid test.

11.7 Neutralization Confirmation

11.7.1 Expose uninoculated carriers to the test substance use solution as in test. 10% of the number of carriers tested during the efficacy portion should be used for each test system.

11.7.2 Transfer carriers to subculture medium after exposure period.

11.7.3 Dilute the test system culture using PBDW to achieve approximately 100 – 1000 CFU/mL. Confirm the number of cells in the suspension by plating 1.0 mL and 0.1 mL of test organism in duplicate utilizing the pour or spread plate technique.

11.7.4 Immediately following the transfers, inoculate the tubes containing the carriers with 0.1 mL of the approximately 100 - 1000 CFU/mL suspension.

11.7.5 Neutralization is confirmed (and valid) when there is growth in all of the tubes inoculated.

**ALTERNATE NEUTRALIZATION METHOD**

Following incubation, randomly select at least one negative tube for each ten tubes tested. Dilute a 24 - 48 hour culture of the test system using PBDW to achieve 100 – 1000 CFU/mL. For fungi, use the conidial suspension used in the test and dilute to achieve 100 – 1000 CFU/mL. Add 0.1 mL of diluted suspension or diluted conidial suspension to each tube. Confirm number of cells in the suspension by plating 1.0 mL and 0.1 mL of test organism in duplicate utilizing the pour or spread plate technique.

11.8 Test System Purity

11.8.1 Streak the test system onto Tryptic Soy Agar with 5% Sheep's Blood or other appropriate non-selective medium. After incubation, observe for purity and perform a Gram stain, if applicable.

## 12.0 OBSERVATION OF SUBCULTURE TUBES & RECORDING RESULTS

12.1 Observe each tube after incubation for absence or presence of organism growth. Growth is indicated by turbidity.

12.2 Record results as number of negative tubes/number of tubes tested.

12.3 If growth is detected, follow the verification procedure as outlined in section 13.0.

12.4 The EPA performance standard for a disinfectant requires the product to kill the test organisms on 59 out of 60 carries (or ten out of ten for *T. mentagrophytes* and *A. niger*). This standard is listed in the Pesticide Assessment Guidelines Subdivision G: *Product Performance*, USEPA 11/82, Series 91.

## 13.0 VERIFICATION OF TEST SYSTEM IN POSITIVE SUBCULTURE TUBES

13.1 Subculture all positive tubes to appropriate medium for the test system (see below) and incubate as appropriate.

13.2 The colony morphology should demonstrate typical characteristics as described below:

- *Staphylococcus aureus* ATCC 6538
  - TSA and Mannitol Salt Agar (MSA)
  - Large yellow colonies are present with yellow zone on MSA indicates *Staphylococcus aureus*
- *Pseudomonas aeruginosa* ATCC 15442
  - TSA and Pseudosel Agar (PA)
  - Fluorescent blue-green colony with a grape-like odor on PA indicates *Pseudomonas aeruginosa*
- *Salmonella enterica* ATCC 10708
  - TSA and MacConkey Agar (MAC)
  - Clear colony indicates non-lactose fermenting gram negative bacilli on MAC, such as *Salmonella enterica*

**TITLE:** **Germicidal Spray Products as Disinfectants**

**NUMBER:** **MS010-21**

- *Trichophyton mentagrophytes* ATCC 9533
  - SAB Agar
  - White downy growth, yellow/white reverse
- *Aspergillus niger* ATCC 6275
  - SAB Agar
  - Black powdery growth, light reverse

Growth on a nutrient medium, plated for each positive subculture tube, allows for further identification beyond that from the differential medium. Perform a Gram stain if further identification is necessary.

13.3 If contamination is present when verifying the positive subculture tubes, the tube containing the contaminant is considered negative for growth of that specific test system. The results would then be changed to reflect this.

## 14.0 RELATED FORMS

14.1 Form 3069: Germicidal Spray Products as Disinfectants

## 15.0 REFERENCES

15.1 2009. 6.3.04 Germicidal Spray Products as Disinfectants, 961.02, Chapter 6 Disinfectants. Official Methods of Analysis of the Association of Official Analytical Chemists
15.2 EPA Good Laboratory Practice Standards, 40 CFR Part 160
15.3 US EPA OCSPP 810.2200 Disinfectants for Use on Hard Surfaces – Efficacy Data Recommendations, March 12, 2012
15.5 MS002: Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims
15.6 MS008: Synthetic Hard Water Preparation & Standardization
15.7 MS040: Media Preparation & Storage – Media & Chemicals

## 16.0 MOST RECENT REVISION SUMMARY

Revised 11.2.1 to include a minimum of three carriers for carrier enumeration for each set dried. Note added to 11.2.2. Adjusted sonication time in 11.2.3. Added 15.3 reference in section 15.0. Removed DIS/TSS-1, DIS/TSS-2 and DIS/TSS-5 references.

Prepared by: Amanda Holen Date: 8/14/12 8/14/12

Quality Assurance: Sarah K Bionstad Date: 15 AUG 2012

Management: Mary Bes Date: 15 Aug 2012

**ECOLAB**
**MICROBIOLOGICAL SERVICES**

**TITLE:** **Test Substance Use-Solution Preparation for Analysis**

**NUMBER:** **MS088-18**

**EFFECTIVE:** **07/02/12**

## 1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration corresponds to the dilution made for the claimed active ingredient concentration in the undiluted test substance.

## 2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows:

2.1.1 Use-solutions prepared at the Lower Certified Limit (LCL) are for efficacy studies and Chemical Quality Verification (CQV) of the use-solution for efficacy studies of EPA regulated products

2.1.2 Use-solutions prepared at the Upper Certified Limit (UCL) are for contract lab TOX studies and CQV of the use-solution for contract lab TOX studies of EPA regulated products

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

2.4 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.3. This use-solution should be labeled according to M032.

**Example:** A 1:64 dilution is 1 part test substance, 63 parts diluent.

2.5 Analyze the test substance for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

**TITLE: Test Substance Use-Solution Preparation for Analysis**

**NUMBER: MS088-18**

**Note:** The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

2.6 Analyze the results. The active ingredient concentration in the use-solution should correspond to the dilution made for the claimed active ingredient concentration in the concentrate (e.g. EPA Upper & Lower Certified Limits) and to 40 CFR § 158.350 Certified Limits unless otherwise noted in the protocol. A scientific explanation must accompany any result which does not correspond to the dilution made for the claimed active ingredient level in the concentrate.

## 3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1. Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume ($DF_{vol}$)

**Example:** Dilution Factor ($DF_{vol}$) $= \left(\frac{1\,oz}{1\,gallon}\right)\left(\frac{1\,gallon}{128\,oz}\right) = 0.0078$

3.1.2 Density/Specific Gravity (SG) Calculation

Obtain density or specific gravity values from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

**Conversion Example:**

$$\left(\frac{9.2\,lbs}{gallon}\right)\left(\frac{1\,gallon}{3785.412\,mL}\right)\left(\frac{453.5924\,g}{1\,lb}\right) = 1.102\,g/mL$$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}};$$

$$\text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

Density of Product = 9.2 lbs/gal ~ 1.102 g/mL;

$$\text{Specific Gravity} = \frac{1.102\,g/mL}{1.0\,g/mL} = 1.102$$

3.1.3 $DF = DF_{vol} \times SG$

$DF = 0.0078 \times 1.102 = 0.0086$

**TITLE: Test Substance Use-Solution Preparation for Analysis**

**NUMBER: MS088-18**

3.2. Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution)

3.2.1 Target mass (g) of product = [Total use-solution mass (g)] ×DF

Target mass (g) of product = 1000 g × 0.0086 = 8.6 g

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Target mass (g) of diluent = 1000 g – 8.6 g = 991.4 g

3.2.3 Include a range of ± 0.03 g (~ 1 drop) or ± 0.3 g (~ 10 drops) to target masses when preparing use-solutions.

**Note:** any appropriate total use-solution mass may be used.

3.3. Use-solution prepared at CSF lower certified limit (LCL) – 1 active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

**Example:** 1 oz/gallon

$$\%\ \text{Dilution} = \left(\frac{1\ \text{oz Product}}{1\ \text{gallon}}\right)\left(\frac{1\ \text{gallons}}{128\ \text{oz}}\right)(100\%) = 0.781\%$$

$$\text{ppm active at LCL} = \left(\frac{\%\ \text{Active at LCL}}{100\%}\right)\left(\frac{\%\ \text{Dilution}}{100\%}\right)\left(\text{Specific Gravity} \times 10^6\right)$$

$$\text{Target mass (g) of product} = \frac{\text{ppm Active at LCL} \times \text{Total mass of use - solution} \times 100\%}{10^6 \times (\%\ \text{Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

**Note:** any appropriate total use-solution mass may be used.

3.4. Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower acceptance limit.

3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

**Note:** any appropriate total use-solution mass may be used.

3.5. Use-solution prepared at CSF upper certified limit (UCL) – 1 active ingredient

3.5.1 Follow 3.3 and replace LCL values with UCL values.

**Note:** any appropriate total use-solution mass may be used.

3.5.2 A use-solution can be purposefully prepared greater than the calculated ppm at UCL concentration.

3.6. Use-solution prepared at CSF upper certified limit (UCL) – multiple active ingredients

- Ensure that all active ingredients are at or above the calculated upper acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount that ensures any active ingredient present to be greater than or equal to the calculated upper acceptance limit.

3.6.1 Follow calculations in 3.5 (replace LCL values with UCL values) to determine target masses (g) of product and diluent.

**Note:** any appropriate total use-solution mass may be used.

3.6.2 A use-solution with multiple actives can be purposefully prepared greater than the calculated ppm at UCL concentration.

3.7. Acceptance criteria formulas and calculations for LCL and UCL dilution use-solutions

3.7.1 **Example:** Product diluted at 1 oz/gallon (product/diluent) for LCL dilution use-solutions

Where: CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Lower Acceptance Level = CSF LCL × DF = 16.43% × 0.0086 = 0.141%

When the analyte of interest in the use-solution at the lower acceptance limit is ≤ 1.0% after dilution; acceptance criteria may be expanded to accommodate method variability or other suitable rationale. Expanded

**TITLE:** **Test Substance Use-Solution Preparation for Analysis**

**NUMBER:** **MS088-18**

*ranges are based on 40 CFR § 158.350 (Certified Limits) for LCL dilution use-solutions.*

| If the nominal concentration (N) for the ingredient is | Upper/Lower Acceptance Limits after dilution may be adjusted as follows | |
|---|---|---|
| | Upper Limit | Lower Limit |
| N ≤ 1.0% | N + 10% | N - 10% |
| 1.0% < N ≤ 20.0% | N + 5% | N - 5% |
| 20.0% < N ≤ 100.0% | N + 3% | N - 3% |

Therefore

Lower Acceptance Limit = 0.141% - 10% → [0.141% – (0.141 × 0.1)] = 0.127%
Upper Acceptance Limit = 0.141% + 10% → [0.141% + (0.141 × 0.1)] = 0.155%

Products with CSF LCL/UCL values greater than N ± 10% should follow the same range as calculated from the CSF.

**Example**

Lower Acceptance Limit = 0.141% - 25% → [0.141% – (0.141 × 0.25)] = 0.106%
Upper Acceptance Limit = 0.141% + 25% → [0.141% + (0.141 × 0.25)] = 0.176%

3.7.2 Example: Product diluted at 1 oz/gallon (product/diluent) diluted at UCL.

Where CSF UCL = 18.15%;DF = 0.0086;N = 17.29%

Upper Acceptance Limit = (CSF UCL × DF) = 18.15 × 0.0086 = 0.156%

The acceptance criteria may be expanded around the UCL dilution. The acceptance range may be increased by 20% and decreased by 10% .

Lower Acceptance Limit = (CSF UCL × DF) – 10% → [0.156 – (0.156 × 0.1)] = 0.140%
Upper Acceptance Limit = (CSF UCL × DF) + 20% → [0.156 – (0.156 × 0.2)] = 0.187%

The acceptance criteria range may be adjusted based on protocol criteria and suitable rationale.

3.8. Acceptance criteria formulas and calculations for use-solutions diluted to the CSF LCL or UCL.

3.8.1 **Example:** Product diluted to 1 oz/gallon

Acceptance criteria for use-solutions diluted to the CSF LCL or UCL are greater than or equal to the Upper/Lower acceptance limits.

JH 9/10/12

**TITLE:** **Test Substance Use-Solution Preparation for Analysis**

**NUMBER:** **MS088-18**

Acceptance Limit (Active at CSF LCL) = CSF LCL × DF = 16.43% × 0.0086 = 0.141%
Acceptance Limit (Active at CSF UCL) = CSF UCL × DF = 18.15% × 0.0086 = 0.156%

Therefore

Acceptance Criteria (Active at CSF LCL) ≤ 0.141%
Acceptance Criteria (Active at CSF UCL) ≥ 0.156%

## 4.0 RELATED FORMS

4.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

## 5.0 REFERENCES

5.1 M032: Labeling Requirements
5.2 40 CFR 158.350

## 6.0 MOST RECENT REVISION SUMMARY

Deleted option of preparing use-solution at label and all corresponding sections. Added CQV use-solution to 2.1.1 and 2.2.2. Added new 3.5.2 and 3.6.2 to allow use-solution to be prepared above calculated ppm at UCL. Revised 3.7.1 to give criteria formulas for use-solutions at LCL. Added 3.7.2 for criteria for use-solutions prepared at the UCL.

Prepared by: Lisa Hellickson Date: 14 June 2013
Quality Assurance: Sherri St. Clair Date: 14 Jun 2012
Management: Mary Beth Date: 14 Jun 2012
6/19/12

**ECOLAB**
**MICROBIOLOGICAL SERVICES**

5

**TITLE:** **Antibiotic Susceptibility Tests**

**NUMBER:** **MS111-04**

**EFFECTIVE:** **09/01/11**

## 1.0 PURPOSE

To verify the antibiotic susceptibility data of test systems. Test systems exhibiting resistant or intermediate antibiotic susceptibility should be confirmed prior to or in conjunction with culture maintenance storage and during the course of a study in which an antibiotic resistant or intermediate test system is tested.

## 2.0 CULTURE MEDIA

2.1 AOAC Nutrient Broth
2.2 AOAC Synthetic Nutrient Broth
2.3 Brain Heart Infusion Broth
2.4 Other medium suitable for culturing specified test systems

## 3.0 SUBCULTURE MEDIA

3.1 Mueller Hinton Broth
3.2 Mueller Hinton Agar
3.3 Tryptic Soy Agar with 5% Sheep's Blood
3.4 Tryptic Soy Agar
3.5 Other medium suitable for culturing specified test systems

## 4.0 REAGENTS & APPRATUS

4.1 0.85% Sterile Saline
4.2 Antibiotics (Oxacillin, Cefoxitin, Vancomycin, etc.)
4.3 Sterile Test Tubes 13 x 100 mm (or equivalent)
4.4 Antibiotic Susceptibility Disks
4.5 Antibiotic Susceptibility Disk Dispensers (optional)
4.6 Sterile Disposable Petri Dishes, 15 × 100 mm
4.7 Transfer Loops: Suitable metal or plastic disposable transfer loops
4.8 Vortex Mixer
4.9 Pipets

## 5.0 TEST SYSTEM PREPARATION

5.1 A minimum of three consecutive transfers but less than 15 total transfers in the appropriate broth medium need to be made before using to inoculate for testing.

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

5.2 If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers.

5.3 If two or more transfers are missed, repeat with three consecutive transfers.

5.4 Transfers can be made on a daily, two day or other appropriate schedule depending on the growth requirements of the test system. Incubate at a temperature that provides good growth.

5.5 From the third or greater consecutive transfer, inoculate Tryptic Soy Agar with 5% Sheep's Blood (BAP) or other non-selective agar medium with a 24 ± 4 hour culture.

## 6.0 INOCULUM PREPARATION

6.1 Turbidity Standard for Inoculum Preparation

6.1.1 To prepare a 0.5 McFarland Standard, add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 mL of 0.18 mol/L (0.36N) $H_2SO_4$ (1% v/v) with constant stirring to maintain a suspension. Verify the density by measuring absorbance at 625 nm. The absorbance should be between 0.08 and 0.13 for a 0.5 McFarland Standard. Store the solution in tightly sealed tubes (same as those used to standardize the bacterial inoculum) in the dark at room temperature. Re-check the density monthly.

6.1.2 Alternately, a 0.5 McFarland Standard may be purchased from a lab supply company.

6.1.3 Measuring the absorbance of the bacterial inoculum at 625 nm in a spectrophotometer is another acceptable method of obtaining an inoculum of 0.5 McFarland Standard. The absorbance should be between 0.08 and 0.13.

6.2 Direct Colony Suspension Method

6.2.1 Inoculate sterile 0.85% saline or sterile broth with isolated colonies from a 24 ± 4 hour incubated non-selective agar plate (refer to 5.5).

6.2.2 Adjust the suspension to achieve a turbidity equivalent to a 0.5 McFarland Turbidity Standard or equivalent. This should result in a suspension containing approximately 1 x $10^8$ to 2 x $10^8$ CFU/mL.

6.2.3 To measure the turbidity of the bacterial inoculum, use either a spectrophotometer (refer to 6.1.3) or perform visually by comparing the inoculum tube and the 0.5 McFarland Standard against a white background with contrasting black lines.

9/10/12

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

6.2.4 This method is recommended for testing fastidious organisms and staphylococci for methicillin or oxacillin resistance.

6.3 Growth Method

6.3.1 Select at least three to five isolated colonies of the same morphology from an agar plate. Touch the top of each colony with a sterile loop or swab and transfer into a tube containing 4 – 5 mL of a suitable broth medium.

6.3.2 Incubate the broth cultures at 35 ± 2°C for 2 - 6 hours or until it achieves or exceeds the 0.5 McFarland Standard.

6.3.3 Adjust the turbidity of the broth culture with sterile 0.85% saline or broth to achieve a turbidity equivalent to that of a 0.5 McFarland Standard.

6.3.4 To measure the turbidity of the bacterial inoculum, use either a spectrophotometer (refer to 6.1.3) or perform visually by comparing the inoculum tube and the 0.5 McFarland Standard against a white background with contrasting black lines.

6.3.5 This method may be used when colony growth is difficult to suspend directly and cannot be made smooth. It may also be used for non-fastidious organisms, with the exception of staphylococci, when 24 hour colonies are not available.

## 7.0 DISK DIFFUSION METHOD

7.1 Pour petri dishes with Mueller Hinton agar or other appropriate medium. Plates should be poured to 4 mm thickness on a level, horizontal surface. If moisture is present on the surface of the agar, dry the plate in a 35 ± 2°C incubator or in a laminar flow hood at room temperature with the lids cracked until dry (typically 10 – 30 minutes).

7.2 Within 15 minutes of adjusting the turbidity of the inoculum (steps 6.2.3 or 6.3.3), dip sterile cotton swab into the adjusted suspension and remove excess liquid from the swab.

7.3 Inoculate the dried agar plate by streaking the swab over the entire agar surface of the plate. Rotate the plate approximately 60° and steak the swab over the entire agar surface. Rotate another 60° and streak with the swab again. Finally, swab the rim of the agar.

7.4 Allow any excess surface moisture on the agar plate to dry. This may be done by leaving the lid of the plate ajar for three to five minutes in a laminar flow hood. The agar plates should dry for no more than 15 minutes.

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

7.5 Dispense the antibiotic susceptibility disks onto the inoculated surface of the agar. The disks should be gently tapped to ensure contact is made with the surface of the agar. Disks should be placed at least 24 mm from each other (center of one disk to the center of another disk) and no more than five disks should be used on a 100 mm plate.

7.6 Within 15 minutes of disk application, invert the plates and incubate for 24 ± 4 hours at 35 ± 2°C. Methicillin Resistant Staphylococci should be incubated for at least 24 hours before reading results.

7.7 Interpret the sizes of the zones of inhibition by measuring the zone diameter, and comparing the value to the package insert from the disk manufacturer to determine susceptible, intermediate or resistance.

**Note:** The disk diffusion method should not be used to determine the intermediate susceptibility or resistance of Staphylococcus aureus to vancomycin. Methicillin resistance can be tested with oxacillin or cefoxitin disks.

## 8.0 MINIMUM INHIBITORY CONCENTRATION (MIC) METHOD

8.1 Antibiotic Standard Solution Preparation

8.1.1 Antibiotics in powdered form should be obtained. Acceptable sources for the antibiotics include drug manufacturers, United States Pharmacopoeia or other commercial sources. Acceptable powders will have a label that states the generic drug name, lot number, potency and expiration date.

8.1.2 Use either of the formulae below to determine the amount of powder or diluent needed to prepare a standard solution. The standard solution concentration will typically be at least 1000 µg/mL or ten times the highest concentration to be tested, which ever is greater.

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}$$

or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

8.1.3 At least 100 mg of powder should be used to prepare the standard.

8.1.4 The standard solution may be filter sterilized, however it is not required since microbial contamination of antibiotic standard solutions is rare.

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

8.2 Macrodilution Procedure

8.2.1 Use 13 × 100 mm sterile test tubes.

8.2.2 Prepare test tubes as outlined in the table in 8.2.6. Alternate volumes may be used to create the desired dilutions/concentrations, but test tubes must have at least 1 mL as a final volume.

8.2.3 Within 15 minutes of adjusting the turbidity of the inoculum to 0.5 McFarland Standard (steps 6.2.3 or 6.3.3), dilute the adjusted inoculum 1:150 in sterile broth (Mueller Hinton Broth or other appropriate medium).

8.2.4 Within 15 minutes of step 8.2.3, add 1 mL of the diluted inoculum to each test tube prepared in 8.2.2. There should be approximately $5 \times 10^5$ CFU/mL test system in the test tubes.

8.2.5 Controls should be performed as follows:

- Positive control: Broth medium with 1 mL of diluted inoculum
- Negative control: Un-inoculated broth medium
- Colony count of inoculum suspensions: Results should be approximately $5 \times 10^5$ CFU/mL
- Test system purity: Streak diluted inoculum to appropriate agar medium

8.2.6 Incubate for 16 - 20 hours at 35 ± 2°C. Evaluations for Vancomycin resistance of *Enterococcus* species should be incubated for 24 – 28 hours.

| Antibiotic Solution | | | | | | |
|---|---|---|---|---|---|---|
| Step | Concentration (µg/mL) | Source | Volume (mL) | Volume of Broth (mL) | Final Concentration (µg/mL) | Volume of Inoculum (mL) |
| 1 | 5120 | Stock | 1 | 9 | 512 | 1 |
| 2 | 512 | Step 1 | 1 | 1 | 256 | 1 |
| 3 | 512 | Step 1 | 1 | 3 | 128 | 1 |
| 4 | 512 | Step 1 | 1 | 7 | 64 | 1 |
| 5 | 64 | Step 4 | 1 | 1 | 32 | 1 |
| 6 | 64 | Step 4 | 1 | 3 | 16 | 1 |
| 7 | 64 | Step 4 | 1 | 7 | 8 | 1 |
| 8 | 8 | Step 7 | 1 | 1 | 4 | 1 |
| 9 | 8 | Step 7 | 1 | 3 | 2 | 1 |
| 10 | 8 | Step 7 | 1 | 7 | 1 | 1 |
| 11 | 1 | Step 10 | 1 | 1 | 0.5 | 1 |
| 12 | 1 | Step 10 | 1 | 3 | 0.25 | 1 |
| 13 | 1 | Step 10 | 1 | 7 | 0.125 | 1 |

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

8.3 Results Interpretation

8.3.1 The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the test system in the tubes or microtiter wells detected by the unaided eye. For a test to be considered valid, there must be growth in the positive control tube.

8.3.2 The presence of the test system must be confirmed in the test tube of the highest concentration with growth. The growth should be subcultured to appropriate media, incubated for 24 ± 4 hours at 35 ± 2°C, and Gram stained.

8.3.3 *Staphylococcus aureus* and Oxacillin: Results that are ≥ 4 µg/mL should be classified as resistant, and ≤ 2 µg/mL are susceptible.

8.3.4 *Staphylococcus aureus* and Vancomycin: Results that are ≥ 17 µg/mL should be classified as resistant, 4 - 16 µg/mL have reduced susceptibility (intermediate) and ≤ 2 µg/mL are susceptible.

## 9.0 MODIFIED HODGE TEST TO VERIFY CARBAPENEMASE PRODUCTION

9.1 Prepare a 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922, transferred as described in section 5.0 with a 24 ± 4 hour culture used to make suspension, in broth or 0.85% saline. Then dilute 1:10 in broth or 0.85% saline.

9.2 Inoculate a Mueller Hinton Agar plate with a diluted suspension prepared as described in 7.2 and 7.3.

9.3 Allow the inoculated plate to dry for 3 – 10 minutes.

9.4 Place one ertapenem or meropenem disk on the inoculated agar plate.

9.5 Using a loop or swab, pick 3 – 5 colonies of the test or Quality Control (QC) organism from a 24 ± 4 hour culture on Tryptic Soy Agar with 5% Sheep Blood or other non-selective medium. Streak growth in a straight line from the edge of the disk out. The streak should be at least 20 mm in length.

9.6 Incubate at 35 ± 2°C for 16 – 20 hours.

9.7 Results interpretation

9.7.1 Examine agar plate for enhanced growth around the test or QC organism streak at the intersection of the streak and zone of inhibition. The enhanced growth will have an appearance of a clover leaf shape.

9/10/12

**TITLE: Antibiotic Susceptibility Tests**

**NUMBER: MS111-04**

9.7.2 Enhanced growth around a test or QC organism streak is a positive test for Carbapenemase production. No enhanced growth is negative for Carbapenemase production.

9.7.3 QC organisms are *Klebsiella pneumonia* ATCC BAA-1705: Modified Hodge Test positive and *Klebsiella pneumonia* ATCC FAA-1706: Modified Hodge Test negative.

## 10.0 RELATED FORMS

10.1 Form 3142: Antibiotic Susceptibility Testing – Disk Diffusion Method
10.2 Form 3143: Antibiotic Susceptibility Testing – Minimum Inhibitory Concentration
10.3 Form 3156: Antibiotic Susceptibility Testing – Modified Hodge Test

## 11.0 REFERENCES

11.1 Clinical & Laboratory Standards Institute Method M2-10A: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 10th Edition. January 2009.
11.2 Clinical & Laboratory Standards Institute Method M7-A7: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard 7th Edition. January 2006.
11.3 Clinical & Laboratory Standards Institute Method M100-S20: Performance Standards for Antimicrobial Susceptibility Testing; 20th Informational Supplement. January 2010.

## 12.0 MOST RECENT REVISION SUMMARY

Revised the incubation range in 7.6. Inserted a new section 9.0: Modified Hodge Test. Added 10.3. Updated the references.

Prepared by: _____ Date: 8/10/11
Quality Assurance: _____ Date: 11 AUG 2011
Management: _____ Date: 16 Aug 2011

9/19/12

Form-Ver. 6016-07
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Form Page 1 of 1

**Regulated Study Protocol Amendment**

**Study Title:** Aqualogic Germicidal Spray Disinfection Efficacy at 10 minutes
**Study Number:** 1200077
**Amendment Number:** 1200077-1A
**Amendment Effective Date:** December 12, 2012

**Description of Amendment**

The protocol is being amended to change the CSF Upper Certified Limit for Free Available Chlorine to 0.1031%.

The protocol is also being amended to include the name Hydris as an alternate name for Aqualogic.

**Scientific Basis for Amendment**

The protocol was amended to change the CSF Upper Certified Limit for Free Available Chlorine from 0.1030% to 0.1031% following a change to the CSF.

The protocol was amended to include the alternate name Hydris for clarification purposes.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

☒ *Study Sponsor* ☐ *Divisional Representative* — *Date* 12 DEC 2012

☒ *Study Director* ☐ *Study Monitor* — *Date* 12/12/12

Printed & Verified Initial & Date 12/12/12

Page 1 of 1